

***Cunninghamella clavata* from Brazil: a new record for the western hemisphere**

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ABSTRACT—During a survey of *Mucorales* in Brejo dos Cavalos (a fragment of an Upland Atlantic Forest within the semi-arid region of Northeast Brazil) a specimen of *Cunninghamella clavata* was isolated from soil samples. A detailed description of the specimen and a phylogenetic analysis of its relationship with other *Cunninghamella* species are presented. An identification key for *Cunninghamella* taxa reported from Brazil is also provided.

KEY WORDS—*Mucoromycota*, *Cunninghamellaceae*, LSU rDNA

Introduction

Cunninghamella Matr. (*Cunninghamellaceae* Naumov ex R.K. Benj., *Mucorales* Fr.) comprises species characterized by the production of sporophores with pedicellate unispored sporangia covered with spines (Zheng & Chen 2001). The sporophores are irregularly, verticillately, or pseudoverticillately branched, and the *Mucor*-like zygospores present a reddish-brown wall (Baijal & Mehrotra 1980, Benny 2006). *Cunninghamella* species have a worldwide distribution, and most are saprobes, often found in soil, stored grains, and other organic substrates (Baijal & Mehrotra 1980, Yu et al. 2015). Some species are pathogenic to humans, particularly immunocompromised patients (Hsieh et al. 2013).

Species of *Cunninghamella* have traditionally been distinguished based on morphological features, such as colony color and texture, pattern of sporophore

branching, vesicle shape and size, sporangiola shape, size, and color, and the presence/absence and length of the projections (spines) in the sporangiola (Zheng & Chen 2001). *Cunninghamella* has been monographed by several authors (Alcorn & Yeager 1938, Cutter 1946, Milko & Beljakova 1967, Samson 1969, Baijal & Mehrotra 1980, Lunn & Shipton 1983) based on morphological characteristics. A subsequent monograph by Zheng & Chen (2001) based on morphological characteristics, maximum growth temperatures, mating experiments, and the length of the ITS region (RFLP analysis) recognized twelve species and three varieties: *C. bertholletiae* Stadel, *C. blakesleeana* Lendn., *C. binariae* R.Y. Zheng, *C. clavata*, *C. echinulata* var. *antarctica* (Caretta & Piont.) R.Y. Zheng & G.Q. Chen, *C. echinulata* (Thaxt.) Thaxt. ex Blakeslee var. *echinulata*, *C. echinulata* var. *nodosa* R.Y. Zheng, *C. echinulata* var. *verticillata* (F.S. Paine) R.Y. Zheng & G.Q. Chen, *C. elegans* Lendn., *C. homothallica* Komin. & Tubaki, *C. intermedia* K.B. Deshp. & Mantri, *C. multiverticillata* R.Y. Zheng & G.Q. Chen, *C. phaeospora* Boedijn, *C. septata* R.Y. Zheng, and *C. vesiculosa* P.C. Misra. Liu et al. (2001) and Yu et al. (2015), who sequenced the ITS rDNA and *tef-1α* gene regions of taxa accepted by Zheng & Chen (2001), found a phylogeny that was consistent with Zheng & Chen's taxonomic conclusions. Subsequently, two additional species have been described: *C. bigelovii* Z. Xin et al. from China (Guo et al. 2015) and *C. gigacellularis* A.L. Santiago et al. from Brazil (Santiago et al. 2016).

Cunninghamella clavata has previously been reported only from Guizhou and Yunnan in southwest China, where the holotype and two paratype specimens were collected in 1985–92 (Zheng & Chen 1998). Here we present a detailed description of a *C. clavata* collection isolated from soil samples from an upland forest area in the semiarid region of Brazil. An identification key for *Cunninghamella* taxa recorded in Brazil and results of phylogenetic analysis of *Cunninghamella* LSU rDNA sequences are also provided.

Material & methods

SOIL COLLECTION—Samples of soil were collected in September 2014 in Brazil's Brejo dos Cavalos Upland Atlantic Forest (8°16'S 35°58'W), located in the city of Caruaru in Pernambuco state, 130 km from Recife. The mean annual temperature is 22 °C, with rainfall ranging from 650 to 800 mm per year. The vegetation consists of montane ombrophilous forest, a remnant of the Atlantic Rainforest. The samples were placed in clean plastic bags and stored in Styrofoam boxes for transportation to the laboratory.

ISOLATION, PURIFICATION, AND IDENTIFICATION OF *C. CLAVATA*—Portions of 5 mg of soil were added to Petri dishes containing a wheat germ agar culture medium

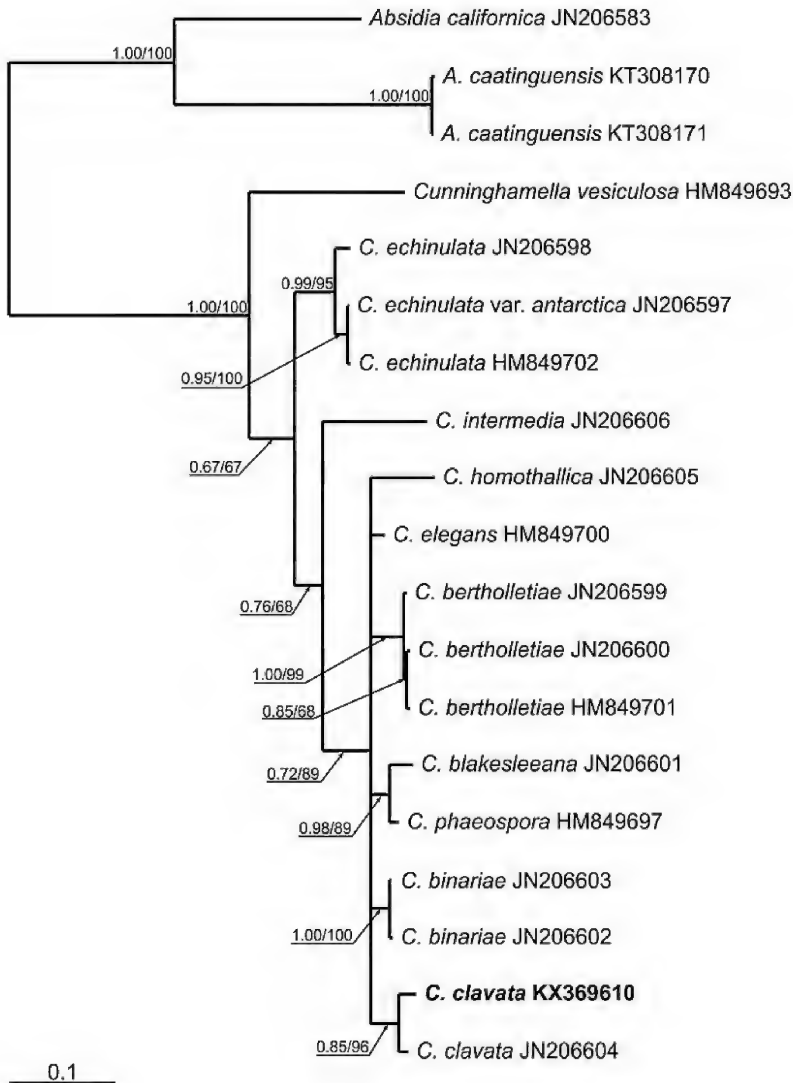


FIG. 1. Phylogenetic tree of *Cunninghamella* taxa, constructed using the large subunit (LSU) rDNA sequences, with *Absidia californica* and *A. caatinguensis* as outgroup. Sequences are labeled with their GenBank accession numbers. Support values are from Bayesian inference and maximum likelihood analyses. The sequence obtained in this study is in boldface.

(Benny 2008) augmented with chloramphenicol (NeoFenicol—Neo Química; 100 mg.L⁻¹). The dishes were stored on the laboratory bench at room temperature (26 ± 2 °C) for seven days and subjected to alternate light and dark periods. Fragments of mycelium were removed from the Petri dishes under a stereomicroscope (Leica EZ4) and transferred to Petri dishes with malt extract agar (MEA) (Benny 2008). The specimen was identified by comparing the macroscopic (colony color, appearance, and diameter) and microscopic (e.g., shape and size of sporophores, columellae, sporangia, and sporangiospores) characteristics with the descriptions of Zheng & Chen (1998, 2001). A voucher was conserved in the Micoteca, Federal University of Pernambuco, Recife, Brazil (URM).

MOLECULAR ANALYSIS—cultures grown in test tubes containing MEA were incubated at 28 °C for six days to obtain fungal biomass. After the material was transferred to 2 mL microtubes with screw caps, 0.5 g acid-washed glass beads of two different diameters (150–212 µm and 425–600 µm, 1:1; Sigma, USA) were added to each tube. The material was crushed through stirring at high speed in a FastPrep homogenizer. The genomic DNA was extracted as described by Góes-Neto et al. (2005). The mycelium was washed with chloroform: isoamyl alcohol (24:1) and then homogenized in 2% cetyltrimethylammonium bromide buffer. The DNA was precipitated in isopropanol, washed with 70% ethanol, and resuspended in 50 µL of ultrapure water.

The primer pairs LR1/LSU2 were used for the amplification of the large subunit (LSU) of nuclear ribosomal DNA (rDNA) (Van Tuinen et al. 1998, Santiago et al. 2014). The polymerase chain reactions were carried out as described by Oliveira et al. (2014). The newly obtained sequences were deposited in GenBank database.

Phylogenetic reconstructions were obtained by analyzing the sequence data of the partial LSU rDNA gene. The fungal sequences were aligned with ClustalX (Larkin et al. 2007) and edited with BioEdit (Hall 1999). Prior to phylogenetic analysis, the nucleotide substitution optimal model was estimated using TOPALi 2.5 (Milne et al. 2004). Bayesian inference (two runs over 1 × 10⁶ generations with a burn-in of 2500) and maximum likelihood (with support estimated by a bootstrap analysis with 1000 replicates) analyses were performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) and PhyML (Guindon & Gascuel 2003), launched from TOPALi 2.5.

Phylogenetic analysis

In BLASTn analysis the *C. clavata* sequence from our collection (URM 7410, KX369610) showed 98% identity with the sequence of the *C. clavata* type culture (CBS 100178, JN206604); these two sequences nested together in the phylogenetic tree (FIG. 1).

Taxonomy

Cunninghamella clavata R.Y. Zheng & G.Q. Chen, Mycotaxon 69: 189 (1998) FIG. 2
COLONY initially white; after 4 days in MEA at 25 °C becoming pale

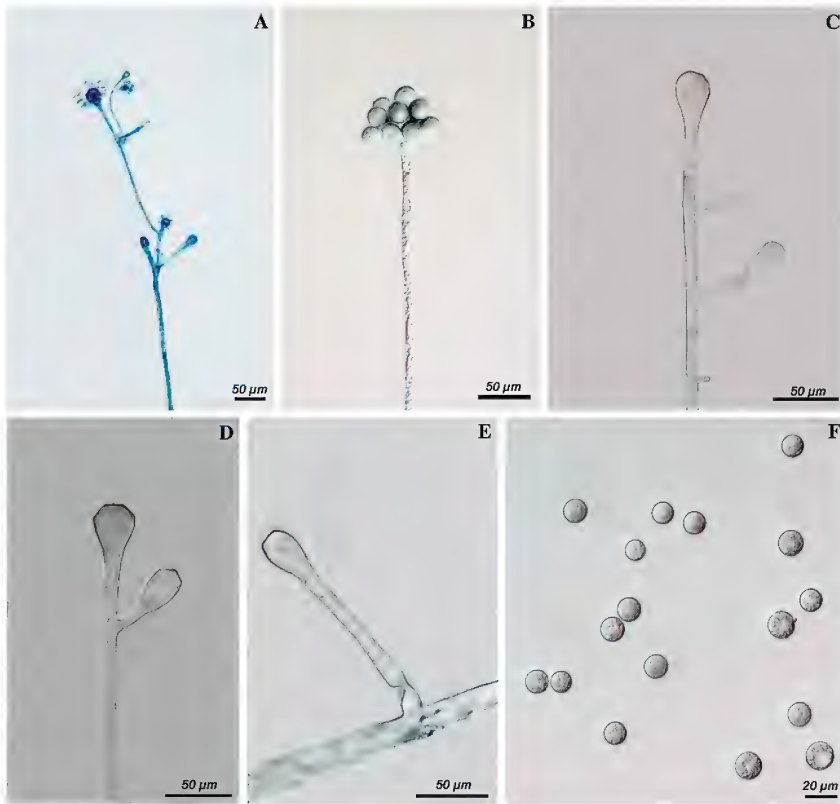


FIG. 2. *Cunninghamella clavata* (URM 7410): A. Repeatedly branched sporophore; B. Sporophore simple with sporangiola; C, D. Sporophores with lateral branches and vesicles; E. Unbranched sporophore with a vesicle; F. Sporangiola.

grayish with cream reverse and reaching 9.4 cm diam. and 9 mm high. RHIZOIDS abundant, long and short, simple or slightly branched. STOLONS present, coenocytic, some showing septa near the sporophore origin. SPOROPHORES short and long, erect or recumbent, arising from stolons or directly from aerial hyphae, hyaline, smooth-walled; some containing one or more septa below the sporangium or near the basis. Main sporophore axes simple or branched, usually equal in width throughout, some enlarging in the apical direction and ending in a vesicle (3.5–)5–10(–14) µm diam.; swelling below the vesicle present. Primary branches simple, in pairs,

pseudo-verticillate (≤ 4). Short and long branches in the same sporophore common, repeatedly ($\leq 6\times$) branching, some arising close to the vesicles and growing parallel to the main sporophores $\leq 500 \times 7.5 \mu\text{m}$, erect or slightly curved and terminating in a vesicle. VESICLES of the main sporophores light gray, mostly clavate, commonly irregular, angular, or flattened at the apex, rarely globose or subglobose, $17\text{--}47.5 \times 20\text{--}37.5 \mu\text{m}$. LATERAL VESICLES with same shape as the main vesicles, $(2.5\text{--})7.5\text{--}35 \times (2.5)5.5\text{--}20(\text{--}25) \mu\text{m}$. SPORANGIOLA globose, light yellow with greenish yellow content and echinulate, $(6.5\text{--})10\text{--}20(\text{--}23) \mu\text{m}$ diam. SPORANGIOSPORES hyaline, echinulate-walled, usually remaining within the sporangiola. CHLAMYDOSPORES and ZYGOSPORANGIA not observed.

HABITAT: isolated from Upland Atlantic Forest soil from the semiarid region of Pernambuco, northeastern Brazil.

SPECIMEN EXAMINED: BRAZIL, PERNAMBUCO, Caruaru: Brejo dos Cavalos, ($8^{\circ}16'S$ $35^{\circ}58'W$), in soil, 29.01.2015, leg. A.L. Alves (URM 7410; GenBank KX369610).

GEOGRAPHIC DISTRIBUTION: Guizhou and Yunnan, China (Zheng & Chen 1998) and Pernambuco, Brazil. This is the first report of *C. clavata* in the western hemisphere.

Discussion

Álvarez et al. (2011) and Walther et al. (2013) noted that the LSU rDNA region is a reliable indication of taxonomic differentiation in *Mucorales*. Our phylogenetic and morphological analyses support *C. clavata* as distinct from the other described *Cunninghamella* species. Morphologically, *C. clavata* is distinguished by producing clavate vesicles and the formation of repeatedly branching short and long branches within the same sporophore—some arising close to the vesicles and growing parallel to the main sporophores (Zheng & Chen 1998).

Cunninghamella echinulata var. *echinulata* also presents clavate vesicles, but lacks the exclusively globose sporangiola observed in *C. clavata*, and in *C. echinulata* var. *echinulata* colonies are white to pale, not pale grayish as in *C. clavata* (Zheng & Chen 2001).

Cunninghamella intermedia also produces strictly globose sporangiola, but its sporophores are not repeatedly branched and the vesicles are globose to subglobose, never clavate. The phylogenetic analysis of the LSU rDNA region confirmed the identity of our specimen.

Our Brazilian specimen represents the first western hemisphere record of *C. clavata* previously reported only from southwest China (Zheng & Chen

1998), contributing to our knowledge of *Mucorales* distribution. In Brazil, other species of *Cunninghamella* such as *C. bertholletiae*, *C. blakesleeana*, *C. echinulata* var. *echinulata*, *C. echinulata* var. *verticillata*, *C. elegans*, and *C. phaeospora* have been isolated from soil and from herbivore dung (Trufem 1981; Santiago et al. 2011, 2013).

Key to *Cunninghamella* taxa registered in Brazil

1. Colonies white to pale or turning yellowish;
giant dark sporangia present or not 2
1. Colonies pale grayish to deep or brownish gray;
giant dark sporangia not present 4
2. Colonies floccose, pale yellowish; sporangia ovoid or ellipsoid ($\leq 30 \times 15 \mu\text{m}$)
or globose ($\leq 17 \mu\text{m}$ diam.); giant dark sporangia not present;
maximum growth temperature 38°C *C. blakesleeana*
2. Colony floccose or granulate, white to pale; sporangia $< 22 \times 18 \mu\text{m}$;
giant dark sporangia present; maximum growth temperature 42°C 3
3. Colony floccose; sporophores usually branched (pseudo)verticillately
with shorter branches on the same sporophore rarely re-branching
..... *C. echinulata* var. *verticillata*
3. Colony granulate; sporophores never (pseudo)verticillately branched;
with shorter branches on the same sporophore often re-branching
..... *C. echinulata* var. *echinulata*
4. Main sporophore axis terminating in a vesicle; long and short branches
on the same sporophore present or absent, if present re-branching $\leq 6\times$ 5
4. Sporophores terminating in a vesicle or dividing into apical branches;
long and short branches in the same sporophore common,
re-branching $\leq 3\times$ *C. bertholletiae*
5. Vesicles clavate; short and long branches in the same sporophore
repeatedly branching $\leq 6\times$ *C. clavata*
5. Vesicles globose, subglobose, ovoid or slightly angular;
sporophore branches rare or common; when present branches
similar in size and rarely re-branching 6
6. Sporophores infrequent, poorly branched;
sporangia brownish to dark brown *C. phaeospora*
6. Sporophores common, well branched;
sporangia grayish to brownish *C. elegans*

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